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Association of leaf micro-morphological characters with powdery mildew resistance in field-grown mulberry (*Morus* spp.) germplasm

Soumen Chattopadhyay[1,4]*, Kabiul Akhter Ali[1], S. Gandhi Doss[1], Nirvan K. Das[1], Ramesh K. Aggarwal[2], Tapas K. Bandopadhyay[3], A. Sarkar[1] and A. K. Bajpai[1]

[1] Central Sericultural Research and Training Institute, Central Silk board, Berhampore 742 101, India
[2] Centre for Cellular and Molecular Biology, Hyderabad 500 007, India
[3] Department of Molecular Biology and Biotechnology, University of Kalyani, Kalyani 741235, Nadia, India
[4] Present address: Regional Muga Research Station, Central Silk Board, Boko 781 123, Assam, India

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Abstract

Background and aims Micro-morphological characteristics can influence fungal infectivity. We sought links between micro-morphology and resistance to powdery mildew in mulberry with the intention of assisting selection of disease-resistant lines.

Methodology Over 3 years and under field conditions, we evaluated 30 lines of mulberry with contrasting susceptibilities to powdery mildew (15 resistant and 15 susceptible). Disease severity was related statistically to stomatal area, stomatal density, stomatal index, upper and lower cuticular thicknesses, leaf thickness and trichome density.

Principal results Differences between lines were significant ($P < 0.05$) for all characters studied. Variation between the resistant and susceptible groups was statistically highly significant ($P < 0.01$) for stomatal index, stomatal area and trichome density. The powdery mildew-resistant group was distinguished by 17.4 % lower stomatal density, 12.5 % smaller stomatal index per unit leaf area, 20.0 % greater trichome density and 18.0 % higher stomatal area compared with the susceptible group. Trichome density was negatively correlated with disease severity index and with the accumulative area under disease progression curves. Stomatal density was positively correlated with both measures of disease severity. Although stomatal area was negatively related to disease severity index ($r = -0.28$; $P < 0.05$), the correlation was weak. There was no statistically significant relationship between stomatal area and the accumulative area under disease progression curves. The germplasm was partitioned into seven sub-groups based on hierarchical cluster analysis derived from pooled disease severity index scores and three highly significant micro-morphological characters. Eighty per cent of the resistant germplasm accumulated in three cluster components (A1, A2 and B2) characterized by high trichome densities and a high stomatal density and stomatal index.

* Corresponding author's e-mail address: soumenchatto@rediffmail.com

Conclusions Resistance to powdery mildew in mulberry is associated with trichome and stomatal features rather than leaf and epidermal thicknesses. Trichome density, stomatal density and stomatal index are shown to be promising markers for screening powdery mildew resistance in breeding programmes.

Introduction

Mulberry (*Morus* spp.) is cultivated widely in geographical areas that include temperate and subtropical regions of the northern hemisphere and tropical parts of the southern hemisphere. Its main use is as the primary food source for the silkworm *Bombyx mori* (Sharma *et al.* 2000). Powdery mildew, an obligate biotrophic ascomycete fungus [*Phyllactinia corylea* (syn. *P. guttata* syn. *P. moricola*); Itoi *et al.* 1982; Brown 2002; Takamatsu *et al.* 2008], is one of the major diseases of mulberry throughout the world. The disease is characterized by white dust-like mycelia that develop over abaxial leaf surfaces. Heavily infected tissues develop chlorosis and senesce prematurely (Gupta 2001). The resulting foliage loss, typically 20 %, reduces substantially the yield of silkworm cocoons (Manimegalai and Chandramohan 2007). Disease control through inherent plant resistance is desirable since it would reduce dependence on costly fungicides that can damage both the environment and the silkworms themselves (Govindaiah and Gupta 2005). Therefore, from both economic and environmental points of view, the development of resistant cultivars is highly attractive.

In a recent screening programme of 147 individuals of various *Morus* species, 6 % of the germplasm showed powdery mildew resistance under both natural and artificial epiphytotics (Chattopadhyay *et al.* 2010). However, mulberry cultivar development is made slow by high heterozygosity and the time required for trees to mature to flowering. The process may be further delayed by reliance on field observations of disease levels, which can be confounded by year-on-year environmental variations. A faster approach would be to seek dependable markers for disease resistance. Accordingly, we have sought readily scored defence-related traits in leaf micro-morphology. This approach may also benefit long-term improvement in other crops (Kolkman and Kelly 2002; Gabler *et al.* 2003).

Plant defence against pathogen attack is complex, with many local and systemic aspects (Felle *et al.* 2004). The internal anatomy and surface features of the leaves often determine plant resistance to biotrophic pathogen infection (Smith *et al.* 1996). Among such characters, aspects of stomata, cuticle and trichome morphology can influence disease resistance (Niks and Rubiales 2002). Unlike other powdery mildew pathogens, the genus *Phyllactinia* is partly (hemi) endoparasitic (Glawe 2008). The pathogen indirectly penetrates the mesophyll via stomata to form haustoria (Takamatsu *et al.* 2008). Therefore, stomata play an important role for *Phyllactinia* infection of compatible hosts (Braun *et al.* 2002). However, stomata-penetrating pathogens need appropriate cues to locate stomatal pores after they adhere to the leaf surface (O'Connell and Panstruga 2006). Trichomes can act as physical barriers to infection (Martin and Glover 2007) and, in *Uromyces,* this can retard germination on the surface of bean leaves by trapping the spores (Mmbaga *et al.* 1994), thereby reducing the probability of germ tubes reaching the penetration site (Wynn 1976). A high frequency of trichomes can also prevent mycelial penetration and infection of other biotrophic fungi (Shaik 1985). Leaf and cuticular or epidermal thicknesses have also been associated with powdery mildew resistance (Commenil *et al.* 1997). Indeed, biotrophy by powdery mildew is initially dependent upon the morphology of the epidermal surface and the adhesion capacity of pathogen spores to the cuticle/cell wall (Vanacker *et al.* 2000; Zeyen *et al.* 2002). In mulberry, germplasm variation in these micro-morphological attributes has been reported (Biasiolo *et al.* 2004; Banerjee *et al.* 2006). However, relationships between these characters and resistance to powdery mildew have not been explored before in mulberry.

Our objectives were to (i) evaluate powdery mildew resistance of 30 lines of mulberry previously regarded as resistant or susceptible, (ii) quantify several micro-morphological characters of leaves from resistant and susceptible plants and (iii) determine the degree of association between foliar micro-morphological characters and powdery mildew resistance.

Materials and methods

Carefully chosen lines in the form of rooted saplings of *Morus* spp. were evaluated in the experimental garden of Central Sericultural Research and Training Institute in Berhampore, India (19 m a.s.l.; 24°6′N and 88°15′E). Saplings from 30 accessions (15 putatively resistant and 15 putatively susceptible) were planted in an augmented randomized block design with 0.6 m × 0.6 m spacing. Three local susceptible accessions ('Bishnupur-4',

'Matigara Black' and 'C-2016') were placed every six rows in a completely randomized manner. A spreader row of 'Kolitha-3' was placed around all sides to encourage a uniform distribution of mildew conidia in the experimental field. The number of test plants was 14 per accession over two sub-plots separated by a spreader row. Three rounds of powdery mildew screening were conducted during October–November (peak season of disease incidence in West Bengal) spanning 2006–2008. The timing coincided with the 'Agrahyani' commercial silkworm rearing schedule of West Bengal.

Disease assessments

Ten plants of each line were scored visually for percentage of leaf area covered by powdery mildew on a 0 (resistant) to 10 (susceptible) scale four times between 32 and 62 days after ground-level pruning in each year. For analysis, disease scores were converted using the modified logarithmic scale of Horsfall–Barrett (Horsfall and Cowling 1978). The scale was 0 = 0 %, 1 = 0–3 %, 2 = +3–6 %, 3 = +6–12 %, 4 = +12–25 %, 5 = +25–50 %, 6 = +50–75 %, 7 = +75–88 %, 8 = +88–94 %, 9 = +94–97 % and 10 = +97–100 %. Disease severity index (DSI) was calculated according to Kim *et al.* (2000) using the following formula:

$$\mathrm{DSI} = \frac{\sum \text{Ratings of each plant}}{10 \times \text{Number of plants rated}} \times 100$$

Powdery mildew development over time was assessed as the area under a disease progression curve (AUDPC) according to the formula of Campbell and Madden (1990) as follows:

$$\mathrm{AUDPC} = \sum_{i=1}^{n} \frac{x_i + x_{i+1}}{2}(t_{i+1} - t_i)$$

where n is the number of evaluation times, x_i is the disease intensity at evaluation time i and t_{i+1} is the time between two disease scores.

Foliar anatomical parameters

Foliar parameters were measured on three consecutive occasions. Sixty-two days after pruning to ground level, the fourth and fifth leaves from the tip of shoots from each accession were placed in plastic bags with a wetted filter and removed to the laboratory for stomatal and anatomical studies. Mulberry is hypostomatous, and stomatal features were assessed using impressions of abaxial leaf surfaces taken at the point of maximum leaf width near the mid-vein using colourless nail polish and adhesive transparent cellophane tape. All impressions were fixed on glass slides and examined under a light microscope at ×100 magnification. Stomata were counted and stomatal density (SD) was calculated as the number of stomata per unit leaf area (mm^2). The stomatal index (SI) was calculated according to Ferris and Taylor (1994) using the formula SI (%) = [(stomata)/(total cells + stomata)] × 100. For stomatal area (SA), 20 randomly selected stomata were measured microscopically using an ocular micrometer (Mishra 1997). Stomatal length was defined as the length of the long axis of the area bounded by the outer stomatal ledges; width was the length of the short axis between the edges of the outer ledges. Stomatal area (μm) was calculated for each stoma using the equation $SA = \pi ab$, where a and b are $\frac{1}{2}$ length and $\frac{1}{2}$ width, respectively. We assumed that the opening between outer ledges was a perfect ellipse (Wise *et al.* 2000). Trichomes were counted from five microscopic fields per leaf sample along the mid-vein side using a Diaplan stereo-dissecting microscope (Lietz M-8) and every half field was converted to number of trichomes per square millimetre (Valverde *et al.* 2001).

Anatomical determinations of abaxial and adaxial cuticle thicknesses (CTb and CTd) and leaf thickness (LT) were made on thick transverse cross-sections (ca. 70 μm) at the point of maximum leaf width. Leaf samples were fixed in formalin–acetic acid–ethanol (1:1:18) for 18 h, dehydrated by passing through ethanol grades and solidified into paraffin (melting point 56 °C) blocks according to Vijayan *et al.* (2008). The sections were made by a rotary microtome.

Data analyses

Analysis of variance was performed using Microsoft Excel version 8.0. When F values were significant ($P < 0.05$), Fisher's least significant differences were calculated. Heterogeneity in the variances was observed in the data from disease screening methods. Accordingly, arcsine-square root transformations were applied before analysis. Student's t-tests for paired two-sample means were used to compare resistant and susceptible germplasm groups. Pearson's correlation coefficients were calculated to compare disease ratings with leaf micro-morphological characters for mean values of each germplasm line (Gomez and Gomez 1984). Further, DSI and AUDPC along with promising micro-morphological values were clustered using 'Statistica' version 8.0 software (Statsoft Inc., Tulsa, OK, USA). The Euclidean distance based on the complete amalgamation rule was used to construct a dendrogram using hierarchical clustering. A linkage distance of 40 was arbitrarily chosen to separate the germplasm into seven clusters in the dendrogram (Berdahl *et al.* 1999).

Results

Significant germplasm variance was observed for DSI, disease progression curves (AUDPC) and all micro-morphological parameters measured (Table 1). Germplasm variances for SI, SD and trichome density (TD) were influenced by year of data collection ($P \leq 0.01$), but such variation was non-significant for DSI, AUDPC, SA, CTd, CTb and LT ($P > 0.05$). Germplasm × year effects were also non-significant for all tested parameters ($P > 0.05$). Moreover, variance component estimates for germplasm were greater than estimates for the year and germplasm × year interaction in all parameters except LT.

Mean powdery mildew scores ranged from 1.6 to 43.8 (variation 27.3-fold) for DSI and from 31.1 to 520.2 (16.7-fold) for AUDPC (Table 2). The most mildew-susceptible germplasm 'Philippines' showed 40–51 % of its leaf area to be affected by the last evaluation date. No line was completely immune to powdery mildew. Reactions to powdery mildew of selected lines (resistant and susceptible) were stable across years (data not shown). Irrespective of resistance and susceptible disease reactions, CTb was significantly higher than CTd for all germplasm (Table 2). Significant differences in SA, epidermal thicknesses and LT were observed among germplasm. Even though mean SA and LT of powdery mildew-resistant germplasm were, respectively, 18 and 6.9 % less than those of the susceptible group, the difference between the two groups was statistically non-significant for these two parameters and for epidermal thicknesses. Significant differences in SD, SI and TD were also found among the mulberry germplasm. Unlike for SA, CTb, CTd and LT, the differences between resistant and susceptible germplasm groups (measured by Student's *t*-test) were also significant for these three features. The mean differences of SD, SI and TD in powdery mildew-resistant and mildew-susceptible reaction groups were 117.2, 3.3 and 4.1, respectively. The resistant germplasm had 20 % more TD with 12.5 and 17.4 % less SI and SD than the susceptible germplasm group.

Stomatal index and density, and trichome features, were significantly correlated with disease susceptibility (Table 3) and showed closely similar relationships with the DSI and AUDPC indices used for disease reaction measurements. Trichome density was negatively correlated with DSI ($r = -0.809$; $P \leq 0.01$) and AUDPC ($r = -0.801$; $P \leq 0.01$), while SI and SD were positively correlated with DSI ($r = 0.576$ and 0.624; $P \leq 0.01$) and AUDPC ($r = 0.561$ and 0.651; $P \leq 0.01$), respectively. A moderately positive correlation of SA and LT with DSI ($r = 0.281$ and 0.260; $P \leq 0.05$) was observed. However, such correlations cannot be generalized since they showed a non-significant relationship with AUDPC. Moreover, the foliar anatomical features abaxial epidermal thickness (ETb) and adaxial epidermal thickness (ETd) showed non-significant relationships with both qualitative and quantitative powdery mildew reactions.

The strong association of SD, SI and TD with both disease responsiveness parameters was partitioned further through hierarchical cluster analysis. The clustering did not differ significantly between DSI and AUDPC. Because of the relatively higher correlations of DSI with SD, SI and TD (Table 3), we chose to present the DSI-based cluster result only (Fig. 1). The entries were grouped into two major clusters (A and B) with a wide linkage distance of 610.5. The sub-cluster A was further divided into two sub-groups (A1 and A2). Eight out of the total of 15 resistant germplasm lines were partitioned into A1 and A2 sub-groups. Two germplasm lines ('Vietnam-2' and 'Zimbabwe-3') in A1 had relatively less SD than the other six lines of the major cluster A. Cluster B represented 22 lines divided into five sub-groups (B1–B5). The sub-groups B1 and B2 included seven resistant germplasm lines with similar SD, SI and TD values to susceptible 'RFS-135', 'Mother graft', 'Shrim-5' and 'Mizuzawa'. The remaining three sub-groups (B3–B5) represented 73 % of susceptible germplasm.

Discussion

Numerous constitutional defence mechanisms against powdery mildew infection have been demonstrated (Prats *et al.* 2006; Hockelhoven 2007). However, most studies have been concerned with the interaction of ectoparasitic powdery mildew pathogens with compatible hosts (Genre and Bonfante 2007; Fernandez-Aparicio *et al.* 2009). Our study provides, for the first time in mulberry, a characterization of the variability of leaf micro-morphological parameters in relation to powdery mildew infection. We used DSI and disease progression curves (AUDPC) to assess differences in powdery mildew susceptibility. These parameters are widely used for assessment of powdery mildew disease reactions (Lipps and Madden 1989; Campbell and Madden 1990). The consistent results across 3 years indicated a strong genetic component and a smaller environmental influence on mulberry powdery mildew resistance. Our results are compatible with those of Lillemo *et al.* (2006), where no influence of environmental (=years) variation affected grossly the level of resistance to powdery mildew in bread wheat progeny.

Our present study indicates that there is substantial germplasm variability in the micro-morphological characters we assessed. It also indicates that environmental

Table 1 Analysis of variance of micro-morphological leaf characteristics of 30 lines of mulberry and their responsiveness to powdery mildew. Evaluations were made over 3 years (2006–2008) in a field environment at Berhampore, West Bengal, India.

Source	Mean squares									
	df	DSI[a]	AUDPC[b]	SI[c]	SZ[d]	SD[e]	CTb[f]	CTd[g]	LT[h]	TD[i]
Germplasm	29	1529.3**	19878.9	257.2**	9933.5**	23717.9**	1.748*	0.418*	4322.1**	194.11**
Year	2	0.695^{ns}	910.9^{ns}	29.0	1215.5^{ns}	1125.5^{ns}	0.0161^{ns}	0.012^{ns}	8636.5^{ns}	96.86**
Year × germplasm	58	0.915^{ns}	178.4^{ns}	1.67^{ns}	230.5^{ns}	65.9^{ns}	0.0211^{ns}	0.014^{ns}	3840.9^{ns}	2.54^{ns}

** and * indicate significance at $P < 0.01$ and $P < 0.05$, respectively.
[a]Disease severity index; [b]accumulative area under disease progression; [c]stomatal index; [d]stomatal density (no. mm^{-2}); [e]individual stomatal size (μm^2); [f]abxial and [g]adaxial cuticle thicknesses (μm); [h]leaf thickness (μm); [i]trichome density (no. mm^{-2}).

(=years) variability was important for SI, SD and TD with a magnitude that warrants further examination. In other plants, a role for foliar micro-morphological traits connected with stomata, epidermis and trichomes in the resistance to biotrophic pathogens is widely acknowledged (Lake and Wade 2009). In our study, five out of seven micro-morphological parameters showed significant association with DSI and three with the disease progression curve. These findings are made the more persuasive by being obtained under the natural powdery mildew infection pressures of the field. They suggest a key role as constitutive barriers against the disease in mulberry. In general, the association with disease reactions was more pronounced for stomatal features and TD than for SA or LT. Although some lines in the mildew-susceptible germplasm (e.g. RFS-135, Mother graft, Shrim-5 and Mizuzawa) have a smaller SD, the number of stomata per unit area of leaf surface and SI were positively correlated ($P \leq 0.01$) with powdery mildew resistance. Unlike *Blumeria* spp. and *Uncinula* spp., the mycelium of *Phyllactinia* spp. is hemi-endophytic (Braun 1987) and the hyphae of *P. guttata* enter the leaf mainly through stomata (Klein *et al.* 1998). There are some significant genotypic effects of stomatal frequency on penetration by powdery mildew pathogens (Lima *et al.* 2010). Our findings corroborate the view that the mechanical obstacles of epidermal thickness and waxy deposition could readily be bypassed during haustorial penetration when the surface porosity of the leaves is high (Gabler *et al.* 2003). We confirm that, in mulberry, resistance to powdery mildew infection increases with decreasing SD and stomatal intensity, with an opposite correlation between SA and DSI. In rust fungi, the emerging germ tubes adhere first to the leaf surface; subsequently, they grow and encounter stomata through directional growth (Wynn and Staples 1981), which in turn triggers appressorium formation (Anker and Niks 2001). Directional growth of the germ tube and formation of appressorium are controlled by the stimuli originating from the host (Hoch and Staples 1987). Excessive wax covering at the stomatal guard cell and epicuticular region obscure brown rust and powdery mildew germlings to recognize specific site(s) that normally triggers appressorium formation (Rubiales et al. 1996; Vaz Patto and Niks 2001). The epicuticular wax crystal pattern has been implicated either in directing or in disorienting germ tube growth across leaf surfaces (Jenks and Ashworth 1999; Rubiales *et al.* 2001). Typically, a germ tube length of 50–60 μm is required to reach the nearest stoma, leaving the germling sufficient energy to go on to infect the plant (Rubiales and Niks 1996). However, the *Phyllactinia* germ tube length needed to penetrate the plant

Table 2 Disease severity of micro-morphological leaf characteristics of 30 lines of mulberry with contrasting susceptibility to powdery mildew. Mean values are shown for DSI, area under disease progression curve, SA, cuticular and leaf thicknesses obtained under field conditions at Berhampore, West Bengal, India, over 3 years (2006–2008).

Accession	Name	Species	Origin	DSI[a]	AUDPC[b]	SI[c]	SD[d]	SA[e]	CTb[f]	CTb[g]	LT[h]	TD[i]
EC-493900	Vietnam-2	*M. multicaulis*	Vietnam	1.6	31.8	20.3	426.6	171.7	1.9	0.9	112.6	20.4
EC-493982	Ankara	*Morus* spp.	Turkey	3.9	61.4	18.0	479.3	134.5	2.2	1.2	123.2	21.5
–	*M.multicaulis* (M)	*M. multicaulis*	Indonesia	6.9	38.9	23.8	524.4	171.7	2.4	1.1	126.0	23.4
IC-313791	Nao-khurkul	*Morus* spp.	India	7.3	45.5	26.6	664.1	224.6	3.4	1.5	143.7	20.0
EC-493796	Kenmochi		Japan	9.1	66.5	22.5	606.7	171.2	2.5	0.8	142.7	22.0
–	Multicaulis	*M. multicaulis*	Indonesia	9.5	85.8	25.3	645.1	171.2	1.7	0.8	117.3	21.6
EC-493973	Rotundiloba	*M. rotundiloba*	Myanmar	8.4	55.4	21.6	506.6	173.2	2.7	1.1	136.7	21.5
EC-493895	*M. multicaulis* (B)	*M. multicaulis*	Indonesia	11.6	101.1	24.3	610.7	269.2	2.5	1.0	121.5	21.4
IC-313861	Non-nayapati	*Morus* spp.	India	12.0	94.5	22.1	489.5	151.1	2.4	1.2	127.2	17.1
EC-493819	Rokokyaso	*M. latifolia*	Japan	11.5	112.4	20.4	479.3	212.8	2.1	0.8	123.9	19.2
EC-49352	Thailand lobed	*M. alba*	Thailand	11.6	72.7	25.8	646.8	223.8	2.4	1.3	134.5	23.1
IC-313976	MR-1	*M. sinensis*	India	13.0	118.2	25.4	668.7	151.2	1.7	1.0	118.0	20.3
IC-314137	Laevigata	*M. laevigata*	India	12.9	90.4	24.4	587.0	146.3	1.8	0.9	120.6	20.6
EC-493856	Zimbabwe-3	*Morus* spp.	Zimbabwe	13.3	125.4	18.4	423.5	130.7	2.6	1.2	135.5	13.8
EC-493842	Akagai	*M. bombycis*	Japan	13.7	102.1	25.8	558.2	200.5	2.3	1.0	125.2	21.9
Mean				9.8	80.1	23.0	554.4	179.8	2.3	1.1	127.2	20.5
IC-313694	RFS-135	*M. indica*	India	29.8	269.7	26.6	618.9	132.3	1.6	0.8	120.8	13.2
EC-493798	Shrim-5	*M. alba*	Bangladesh	36.8	390.5	20.8	618.2	165.8	2.2	1.0	120.6	11.6
IC-313733	Kolitha-3	*M. indica*	India	30.4	322.2	34.8	947.4	163.4	1.5	0.6	93.8	12.9
IC-313697	Mysore local	*M. indica*	India	30.0	312.0	31.3	839.8	145.0	2.2	0.9	132.9	11.5
IC-313832	Kurseong	*M. indica*	India	30.8	279.3	33.8	979.3	153.6	2.3	1.0	135.6	12.4
IC-313667	Mother graft	*Morus* spp.	India	30.3	245.6	23.4	629.9	147.0	2.4	0.9	132.6	12.4
EC-493791	Mizuzawa	*M. bombysis*	Japan	32.2	273.5	24.8	618.7	156.9	1.5	0.7	106.6	8.7
IC-313687	Acc 119	*Morus* spp.	India	30.9	242.9	27.5	732.1	126.2	2.0	1.0	112.7	15.0
IC-313821	Tista valley	*M. alba*	India	32.5	333.0	32.3	826.0	155.2	2.1	1.0	107.2	14.0
IC-313692	S-30	*M. indica*	India	31.9	181.1	31.1	734.2	163.5	1.9	1.0	104.1	11.1
EC-493901	Xuan-9	*Morus* spp.	China	37.4	448.0	30.8	931.2	159.4	2.7	1.3	114.1	13.0
IC-313872	Sujanpur local	*M. alba*	India	36.3	378.3	37.7	947.2	121.4	2.5	1.3	121.2	12.2
IC-313820	Kolitha-9	*M. indica*	India	42.6	438.9	37.0	980.9	143.4	2.7	1.2	130.6	10.6
EC-493777	Burma-8	*M. indica*	Myanmar	40.9	445.3	26.5	762.2	127.2	2.3	1.0	112.5	12.3

EC-493768	Philippines	*M. indica*	Philippines	43.8	520.2	22.8	765.3	149.3	3.1	1.3	134.0	13.1
Mean				34.5	338.7	29.5	788.7	147.3	2.2	1.0	118.6	12.3
Grand mean				22.1	209.4	26.3	671.6	163.6	2.3	1.0	122.9	16.4
$LSD_{(0.05)}$				1.74	28.9	2.59	70.7	44.4	0.47	0.33	4.0	4.07
t-value res. vs. sus.				**	**	**	**	*	ns	Ns	ns	**
Cv%				39.8	70.0	21.1	25.5	16.3	7.4	6.7	11.4	29.7

Scores are based on mildew severity ratings of 10 plants per entry per season for three consecutive years made during October–November.
Disease scores are back transformations of arcsine [$\sqrt{(x/100)}$]. DSI was estimated from the obtained scores of 60-day-old plants and grading was done on the basis of DSI values using the 10-point scale of Horsfall–Barrett (Horsfall and Cowling 1978). Area under a disease progression curve was estimated from four scores of disease reactions made between 30 and 60 days per season. Leaves with mean DSI ≤ 12.0 and/or AUDPC ≤ 130.0 and ≥ 25.0 and/or ≥ 200.0 were considered resistant and susceptible, respectively.
[a]Disease severity index; [b]accumulative area under disease progression; [c]stomatal index; [d]stomatal density (no. mm^{-2}); [e]stomatal size (μm^2); [f]abaxial and [g]adaxial cuticle thicknesses (μm); [h]leaf thickness (μm); [i]trichome density (no. mm^{-2}).
– and spp. = unknown accession number and species, respectively.
*, ** and ns indicate significance at $P < 0.05$, $P < 0.01$ and non-significant, respectively.
Anatomical data are means of five observations per microscopic field ($n = 5$) for three consecutive seasons during October–November spanning 3 years.

surface through stomata remains unresolved. Moreover, extensive wax covering of the stomatal guard cells presumably decreases optimum gas exchange through the stomata required for pathogen penetration (Vaz Patto *et al.* 2003). In mulberry, stomata are randomly scattered over the abaxial leaf surface (Tikader and Rao 2002). In *Lolium* spp., *Hordeum chilense* and *Pisum sativum*, abaxial leaf surfaces showed more penetration resistance to leaf rust and powdery mildew conidia, respectively, than adaxial epidermal cells (Carver *et al.* 1990; Rubiales and Carver 2000; Gniwotta *et al.* 2005). The composition of abaxial and adaxial wax is different. The adaxial wax was characterized by a very high amount of primary alcohols, while abaxial wax consisted mainly of alkenes (Gniwotta *et al.* 2005). Variations of cuticular wax (Mamrutha *et al.* 2010) and secondary plant constituents like phenolics and flavonoids (Song *et al.* 2009) are also marked among mulberry accessions. A differential exudation of phenolic compound coumarins (like scopolin, ayapin and scopoletin) in the leaf surface of sunflower genotypes prevents rust germ tube growth and appressorium differentiation (Prats *et al.* 2007). Therefore, in addition to leaf architectural barriers such as epicuticular and/or stomatal guard cell wax content, the possibility of a higher exudation of coumarins onto the leaf surface of resistant lines of mulberry cannot be overruled. Our finding of a significantly smaller SD in powdery mildew-resistant lines compared with the susceptible group supports the possibility that germ tubes could also form misplaced appressoria that are too far from the nearest stoma. This situation has been reported in the case of the faba bean–rust interaction (Sillero and Rubiales 2002). A positive correlation of leaf rust resistance with SD on the abaxial leaf epidermis was reported in *H. chilense* lines and suggested that it might be a provision to compensate for the presumed less efficient gas change by wax-covered stomata (Rubiales and Niks 1996; Vaz Patto *et al.* 2001). But the exact implication of SD in *Phyllactinia* resistance in mulberry would be an area of further study in future. Nonetheless, our observations strongly suggest that stomatal traits can be used effectively in mulberry breeding programmes to predict powdery mildew resistance.

The contrast in TDs between the powdery mildew-resistant and mildew-susceptible germplasm groups was marked. Further, a strong negative correlation between TD and the AUDPC was also established. Our results support those of Shaik (1985), who found that resistance to *Uromyces appendiculatus* infection in beans was attributable to the physical effect of densely packed trichomes. However, the exact role of trichomes in preventing *Phyllactinia* spore penetration into the host leaf is still unknown. It has been reported that

Table 3 Pearson's correlation coefficient (*r*) between powdery mildew DSI or accumulative disease progression and seven micro-morphological characteristics of mulberry leaves. Thirty mulberry lines were evaluated under field conditions at Berhampore, West Bengal, India, over 3 years.

	SI[a]	SA[b]	SD[c]	CTb[d]	CTd[e]	LT[f]	TD[g]
DSI[h]	0.576**	−0.281**	0.624**	0.051^{ns}	0.014^{ns}	−0.260*	−0.809**
AUDPC[i]	0.561**	−0.280*	0.615**	0.094^{ns}	0.005^{ns}	-0.255^{ns}	−0.801**

** and * indicate significance at $P < 0.01$ and $P < 0.05$, respectively.
[a]stomatal index; [b]stomatal area (μm^2); [c]stomatal density (no. mm^{-2}); [d]abaxial and [e]adaxial cuticle thicknesses (μm); [f]leaf thickness (μm); [g]trichome density (no. mm^{-2}); [h]disease severity index; [i]accumulative area under disease progression.

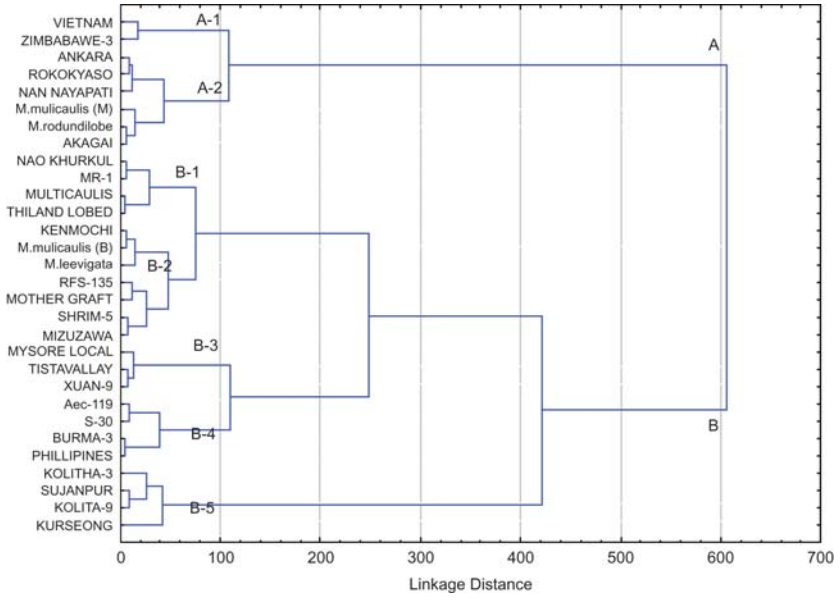

Fig. 1 Dendrogram of 30 mulberry germplasm lines with contrasting degrees of susceptibility to powdery mildew. The relationships were obtained by analysing DSI, AUDPCs, SD, stomatal intensity and TD values during powdery mildew infection.

the conidia of an ectoparasitic powdery mildew pathogen need a liquid droplet to erode the cuticle immediately after contact (Mendgen 1996). From the work of Kortekamp and Zyprian (1999), it appears that an increased number of hydrophobic pubescences (such as trichomes) may repel water from the leaf surfaces, thus preventing successful penetration. Alternatively, a high trichome number may simply reduce the frequency of germ tube contact points that can lead to penetration (Niks and Rubiales 2002).

Both ETb and ETd and LT were very similar in most of the lines we studied. However, variation in cuticle thickness of both adaxial and abaxial surfaces was statistically significant across all 30 lines, but there was no clear difference between susceptible and tolerant groups. Our findings support previous work with *Uncinula nector* resistance in grape berries by Ficke *et al.* (2004), which showed only a weak relationship with cuticle thicknesses and disagree with the report of a positive correlation of cuticle thickness of various grape cultivars resistance to powdery mildew (Heintz and Blaich 1990). The exact reason for the discrepancy in the correlation between LT or SA and disease prevalence is uncertain. However, *Phyllactinia*, being a hemi-endophyte, penetrates the leaf surface through stoma; therefore, cuticle thickness might have little role in penetration resistance. Overall, our results indicate that resistance to powdery mildew is not strongly associated with cuticle thickness and only weakly associated with LT and the area of individual stoma.

The cluster analysis showed good agreement between the DSI and SD, SI and TD. However, three resistant lines

('Kenmochi', '*M. laevigata*' and '*Multicaulis*') did not reveal a sharp demarcation with four other susceptible lines of sub-group B2. The lack of tight clustering of these three lines may indicate that evaluation of other features is necessary to establish a more complete phylogenetic relationship among powdery mildew-responsive accessions.

It seems, for the first time, that an alternative 'avoidance' or pre-penetration mechanism, which operates after the contact of parasite on the host epidermal cell (Rubiales and Niks 1992; Vaz Patto *et al.* 2009) is apparent in mulberry–powdery mildew interaction. In several species, the pre-penetration resistance is often superimposed onto later-acting post-penetration hypersensitive resistance (Niks and Rubiales 2002). Contrary to relatively less durable hypersensitive resistance, pre-penetration avoidance is quantitative trait loci in nature, more durable and therefore has a greater value in breeding work (Vaz Patto *et al.* 2003).

Conclusion and forward look

Highly significant and similarly strong correlations were found between the prevalence of powdery mildew and three micro-morphological parameters (SI, SD and TD) among 30 lines of mulberry with known susceptibility or resistance to the pathogen. The links appeared causal and may be related to the level of successful spore penetration of the leaf. These three micro-morphological traits are thus of potential value when selecting for powdery mildew resistance from progeny or from collected material used in mulberry breeding programmes.

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Contributions by the authors

All the authors contributed to a similar extent to the experimental work. S.C. prepared the manuscript.

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Conflict of interest statement

None declared.

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